Project no.: **037296**

Project acronym: **EUROIRON1**

Project title:

GENETIC CONTROL OF THE PATHOGENESIS OF DISEASES BASED ON IRON ACCUMULATION

Instrument: **SPECIFIC TARGETED RESEARCH PROJECT**

Thematic Priority: **LIFE SCIENCES, GENOMICS AND BIOTECHNOLOGY FOR HEALTH**

Title of report:

Publishable final activity report

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Revision: [FINAL]

A.1 Description of the project objectives

The occurrence of diseases of iron accumulation such as Genetic Iron Overload (GIO) and Anaemia of Chronic Diseases (ACD), and their phenotypic variability, involve genetic abnormalities that affect the expression of proteins involved in iron metabolism.

A.1.1. **The scientific objectives** of this project aimed at: a) elucidating the genetic factors involved, directly or indirectly, with expression of the key genes of iron metabolism associated with pathological iron accumulation ; b) assessing the functional consequences of their abnormal expression in cellular and animal models ; c) exploring how this knowledge can be used for introducing diagnostic and prognostic markers (genetic and functional) for defining the particular type of GIO or ACD concerned and its optimal therapeutic management by established and experimental procedures. In a broader perspective, an objective will be to contribute to the improved knowledge of the genetic control of the pathogenesis of other diseases based on iron metabolism disorders.

A.1.2. **The technological objectives** consisted in the development of innovative methodological approaches and tools, based on animal and cellular models, and resorting to analytical methods for *in situ* tracing of cellular labile iron pool and gene expression studies. Moreover, specific cohorts of patients with haemochromatosis or anaemia of chronic disease were studied.

A.2 Consortium

Role	N°	Participant organisation name	short name	Status	Country
CO*	CR1*	UNIVERSITY OF RENNES 1	UR1	GOV	FRANCE
CR**	CR2	KINGS COLLEGE LONDON	KCL	PRIV	U K
CR	CR3	THE HEBREW UNIVERSITY OF JERUSALEM	HUJI	PRIV	ISRAEL
CR	CR4	UNIVERSITA DEGLI STUDI DI MODENA E REGGIO EMILIA	UNIMORE	GOV	ITALY
CR	CR5	UNIVERSITA VITA-SALUTE SAN RAFFAELE	UHSR	PRIV	ITALY
CR	CR6	UNIVERSTÄTSKLINIKUM HEIDELBERG	UKL-HD	GOV	GERMANY
CR	CR7	EUROPEAN MOLECULAR BIOLOGY LABORATORY	EMBL	GOV	GERMANY
CR	CR8	MEDICAL UNIVERSITY OF INNSBRUCK	IMU	GOV	AUSTRIA
CR	CR9***	INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE	INSERM	GOV	FRANCE
CR	CR10	PARTNERCHIP	PRC	SME	FRANCE
CR	CR11	UNIVERSITY OF BRESCIA	BRE	GOV	ITALY

***CO = Coordinator ;**
****CR = Contractor ;**
***** CR9 : The CR9 partner is composed of two INSERM teams, one in Toulouse and the other one in Paris. In the following detailed scientific programme, they will be identified respectively as CR9a and CR9b.**

A.3 Coordinator Contact Details

Contact of the coordinator:

- Project coordinator name: Prof. Pierre BRISSOT
- Project coordinator organisation name: University of Rennes1
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A.4 Summary of work performed, achievements, end results

A.4.1. Genetic control of cellular iron uptake

. Focusing on intestinal heme iron uptake, so far poorly characterized in contrast of non heme iron uptake, this research has shown that: i) in knock-out mouse models mimicking the genetic iron overload situations of type 1 (HFE-related) and type 2 (hepcidin-related) haemochromatosis, heme iron uptake is also enhanced, therefore contributing to the development of iron overload; ii) competition exists between substrates heme and folate for the transport protein HCP1.

. Using the differentiated HepaRG cell model, an increased expression of L-ferritin gene by spermine and spermidine was found, reflecting at least partly an increased cellular iron storage, probably related to increased iron uptake when considering the amplified expression of the iron transporter DMT1.
. A significant reducing effect of the calcium channel blocker verapamil on tissue iron loading in HPX (hypotransferrinemic) mice, in contrast of hepcidin knock-out mice, suggesting that genetic factors may may modulate the verapamil effect.

A.4.2. Genetic control of cellular iron egress

Two main pathological situations, characterized by decreased cellular iron egress, have been studied: the ferroportin disease and the anaemia of chronic disease (ACD).

A.4.2.1. Ferroportin disease

Studying ferroportin expression in macrophages (circulating monocytes) from patients with ferroportin disease, it was found that, as compared with normal controls and Hfe haemochromatosis patients, ferroportin expression, for all tested mutations, was : i) increased in ferroportin patients' macrophages, ii) predominantly located intracellularly (versus plasma membrane localisation), and iii) not inhibited by hepcidin. These data indicate that ferroportin mutations impair ferroportin trafficking to the plasma membrane and induce iron retention in human monocytes-macrophages. These homogeneous data contrasted with the marked differences observed between various cell lines when testing ferroportin mutants.

A.4.2.2. Anaemia of chronic disease

. Studying ACD and ACD + IDA (iron deficiency anaemia) in a rat model and in patients, low duodenal ferroportin expression was found, together with high serum hepcidin levels, in ACD whereas an opposite situation was observed in ACD + IDA. These data have important clinical applications since hepcidin levels, which are more responsive to the erythropoietic demand for iron than to inflammation, may help to differentiate ACD and ACD + IDA and to select appropriate iron therapy in these patients.

. Using a coculture model, a stimulation of ferroportin expression upon pathogens (Salmonella typhi murium, Aspergillus fumigates) was found. This enhanced ferroportin expression promoted iron export, therefore limiting iron availability for pathogens.

A.4.3. Genetic control of systemic iron regulation (WP3)

A.4.3.1. Determination of hepcidin role in iron regulation

The main achievements regarding hepcidin, known as the master regulator of body iron status in human body, were the following:

. A human monoclonal antibody has been patented. It is of high affinity for hepcidin and speficic to human hepcidin. It recognizes serum human hepcidin and possesses a neutralizing activity on hepcidin in vitro as well as in vivo. Therefore, this antibody should allow developing an ELISA assay for clinical applications.

. In contrast to macrophagic cell types, hepatocytes had been poorly studied in terms of the presence of the iron exporter ferroportin. The present data, obtained in an hepatocyte culture system and in hepcidin-deficient mice, showed that hepatocytic ferroportin is a target of hepcidin. The fact that hepcidin can trigger ferroportin degradation in hepatocytes should be taken into account when considering hepcidin therapeutics.

. The inhibition, through specific SiRNAs, of hepcidin expression in the coculture model associating mouse hepatocytes and rat liver epithelial cells, has provided a list of promising genes that are regulated by hepcidin, independently of iron overload.

. Transcriptomic and proteomic analysis of hepcidin knock-out mice showed a decrease in liver and serum haptoglobin due to failure to fold and assembly proteins in the endoplasmic reticulum affected by the iron related oxidative stress. This result is important knowing the many clinical situations which can induce endoplasmic reticulum stress.

A.4.3.2. Characterization of genes involved in the control of systemic iron regulation

. A new coculture model, combining mouse hepatocytes and rat liver epithelial cells, has been set up. It allows a very high level of hepcidin mRNA expression and therefore represents a valuable tool for studying hepcidin regulation. Natural (curcumin) and synthetic STAT3 inhibitors have been shown, in this model, to reduce hepcidin expression in differentiated mouse hepatocytes expressing the active phosphorylated STAT3 form.

. Accumulation of iron within periportal hepatocytes is a feature of iron overload of digestive origin. Studying, at the transcriptional level, the expression of iron-metabolism genes in mouse hepatocytes, no metabolic lobular zonation was found for most genes, including in particular hepcidin and ferroportin. However, a preferential periportal expression of ceruloplasmin was observed, raising the issue of its special role in iron overload disorders involving a defect in cellular iron export.

. Hemojuvelin is known as a key protein of iron metabolism, leading, when mutated, to juvenile haemochromatosis. Characterisation of hemojuvelin showed that defective targeting to the plasma membrane accounts for the inability of most hemojuvelin mutants to activate hepcidin. Moreover, investigating the origin of soluble hemojuvelin, it has been demonstrated that it is produced by a furin cleavage.

. SMAD7 controls iron metabolism as a potent inhibitor of hepcidin expression.

. BMP6 has been demonstrated as a major gene involved in the control of hepcidin expression as demonstrated by the strong haemochromatosis phenotype of BMP6 -/- mice. This finding is a real breakthrough since BMP6 may constitute a strong candidate to modulate endogenously the production of hepcidin.

. Despite the key implication of BMP6 in hepcidin expression, Hfe -/- mice exhibit an increased BMP6 expression (related to hepatic iron overload) contrasting with decreased hepcidin expression. These data suggest that Hfe contributes to the BMP/SMAD cascade in a way which is critical for the regulation of hepcidin expression.

. Focusing on the novel major hepcidin suppressor, TMPRSS6 (matriptase-2), identified by others in 2008, we demonstrated that matriptase-2 inhibits hepcidin only in the presence of hemojuvelin and by cleaving the hemojuvelin isoform localized on the plasma membrane.

. CREBH (cyclic AMP response element-binding protein H), involved in the endoplasmic stress response, has been identified as a critical factor in the response to hepcidin promoter in stress situations. This opens the new concept that endoplasmic reticulum stress controls iron metabolism through induction of hepcidin.

. A cross-talk between the mitogen activated protein kinase and bone morphogenetic protein/hemojuvelin pathways is required for the induction of hepcidin by holotransferrin in primary mouse hepatocytes.

. HIF-2 alpha, but not HIF-1 alpha, promotes iron absorption in mice, as shown by the fact that specific deletion of HIF-2 alpha leads to decreased serum iron, liver iron and liver hepcidin expression. This finding may provide the basis for development of new strategies to improve iron homeostasis in patients with iron disorders.

. Secondary iron overload in mice leads to decreased plasma iron bioavailability related to hepcidin increase with subsequent limitation of splenic iron release. These data support the importance of managing hepcidin levels before starting venesection therapy in patients that present with secondary iron overload and are eligible for phlebotomies.

A.4.3.3. Validation studies of the targeted genes and pathophysiological mechanisms in humans

. A new mutation in the hepcidin promoter impairs its BMP response (proximal BMP-RE) and contributes to a severe phenotype in Hfe-related haemochromatosis. This finding is of clinical importance in terms of diagnosing unexplained iron overload situations.

. Daily regulation of serum hepcidin is not influenced by submaximal muscle exercise in healthy volunteers, suggesting that muscles may not be physiologically a major modulator of iron homeostasis, and having potential implications for hepcidin sampling in medical practice.

. Studying serum levels of GDF-15, a growth differentiation factor associated to iron metabolism through modulation of hepcidin expression, in patients with ACD, ACD + IDA, and IDA, it was concluded that other ACD-related factors may overcome the regulatory effects of GDF-15 on hepcidin expression during inflammation.

. A novel form of secondary iron overload has been identified. It corresponds to acquired aceruloplasminemia related to copper deficiency, itself due at least partly to excessive zinc intake. These findings underline the strong interactions between zinc, copper and iron metabolisms.

A.4.4. Animal and cellular models and novel technologies

. Generating conditional HFE knock-out mice, the demonstration was made that local HFE expression in hepatocytes serves to maintain physiological iron homeostasis in general and hepcidin expression in particular, answering a longstanding question in medicine.

. Using fluorescent probes, demonstration that the iron chelator deferiprone can act as a siderophore.

. Using frataxin-deficient cells, demonstration that deferiprone-mediated relocation of iron restores cell functions impaired by frataxin deficiency. This finding sheds light on the mechanism whereby deferiprone appeared to be beneficial in patients with Friedreich ataxia.

. Development of animal models involving IRP (Iron Regulatory Proteins) : successful generation of IRP1-/-, IRP2-/- mice revealed that IRE/IRP system prevents mitochondrial iron deficiency thus safeguarding mitochondrial function.

. Development of animal models involving HFE : a) Generating Hfe-/- and double knock-out Hfe/lipocalin-2 mouse models infected by Salmonella typhimurium, HFE deficiency was shown to protect from infection via induction of lipocalin-2. b) Using the model of macrophage specific *Hfe*-/- mice, macrophage HFE was shown to be critical for efficient infection with Salmonella. c) Using a BALB/c mouse model of Mycobacterium avium infection, the developing anaemia was demonstrated to be independent of increased hepcidin expression, possibly involving ferroportin and/or lipocalin.

. Development of cell models involved in the pathogenesis of disorders of iron metabolism. a) Thanks to the hepatocyte-Kupffer cell coculture model, we were able to show that Kupffer cells modulate iron homeostasis in mice via regulation of hepcidin expression. b) The coculture model combining mouse hepatocytes and rat liver epithelial cells proved valuable for studying hepcidin regulation ; c) Using the HepG2 cell line submitted to specific gene silencing, a new mechanism of hepcidin regulation was proposed involving a cross-talk between BMP signalling and the HFE/TfR2 signalling.

. Development of novel technologies for the functional assessment of genes related to iron metabolism. a) Methods for tracking intracellularly the labile iron were consolidated as well the design of agents for attaining iron redistribution. b) The Raw cell macrophage model was developed for studying the role of NTBI-mediated iron overload and of erythrophagocytosis. c) A line of insulin secreting cells showing extreme susceptibility to NTBI loading has been established. Recapitulating the in vivo properties, this endocrine cell model should allow assessment of chelation efficacy.

A.4.5. A systematic approach to identify new genes controlling body iron stores

Genotyping of 120 HFE -/- mice, completed for almost 400 SNP markers, has permitted to identify 8 clusters of genes exhibiting a summary expression trait significantly correlated with iron. For 3 of these clusters, containing each approximately 20 genes of interest, an expression driver was localised on chromosomes 7, 8 or 11, coinciding with the candidate regions controlling liver iron content previously identified in an independent cross.

The phenotypic and genotypic study of a genetic Italian isolate consisting of 1800 subjects has confirmed that the widely reported effect of HFE and TMPRSS6 on haemoglobin and erythrocyte trait variations is mediated by their effect on iron homeostasis. They also suggest that TMPRSS6 variants, opposite to HFE C282Y, are good candidate modifiers of the phenotype of the C282Y/C282Y patients and might contribute to the non-penetrance of HFE-hemochromatosis.

Almost 200 patients with either severe or mild/asymptomatic C282Y homozygotes have been genotyped for 250K SNPs. Preliminary analyses have shown associations between SNPs on different chromosomes and disease severity. Some of them are located in genes that could be functionally relevant for iron metabolism.
These complementary approaches, based on no *a priori* and on the analysis of large series of SNPs covering the whole genome and, in mice, genome-wide expression studies, have therefore led to the identification of a manageable set of genes to test for functional causality.

In conclusion, identifying new physiological actors intervening in iron metabolism, elucidating the mechanisms whereby mutated genes generate iron disorders, and discovering genes which account for the variable phenotypic expression, have constituted the main scientific goals of the present consortium.
The improved understanding of the genetic mechanisms underlying widespread diseases related to iron accumulation, which has been permitted by EuroIron1, should, in our view, lead in the near future to the proposal of novel diagnostic, prognostic and therapeutic approaches which will ameliorate the overall curative and preventive management of these disorders.

A.5 Publishable results for using and disseminating the knowledge

The present project has provided a number of important data.

1. New tools for exploring iron metabolism and its disorders:

. New models both ***in vivo*** (conditional HFE knock-out mice ; hepcidin knock-out mice ; ferroportin knock-in mice ; IRP1-/-, IRP2-/- mice ; HFE/lipocalin-2 knock-out mice ; rat with anemia of chronic disease) and ***in vitro***, providing interesting alternative to in vivo models, such as co-cultures (combining hepatocytes and kupffer cells or mouse hepatocytes and rat liver epithelial cells ; HepG2 cell lines submitted to specific gene silencing).
. New methods for tracking intracellular labile iron with the design of agents for attaining iron distribution.
. Production of a patented human monoclonal hepcidin antibody

2. Identification of new genes of iron metabolism and /or characterization of new regulatory processes for iron metabolism :

. Key implication of BMP6 in hepcidin expression
. Dissection of the mechanism linking TMPRSS6 and hepcidin expression
. Role of Smad7 as an inhibitor of hepcidin expression
. New concept that endoplasmic reticulum stress controls iron metabolism through induction of hepcidin (critical role of CREBH)
. Identification of Atoh8 as being co-regulated with hepcidin by iron burden
. Natural and synthetic STAT3 inhibitors reduce hepcidin expression in the coculture (mouse hepatocytes/rat liver epithelial cells)
. HIF2-alpha promotes iron absorption in mice
. Demonstration that IRE/IRP system safeguardes mitochondrial function by preventing mitochondrial iron deficiency
. Hepcidin can trigger ferroportin degradation in hepatocytes.

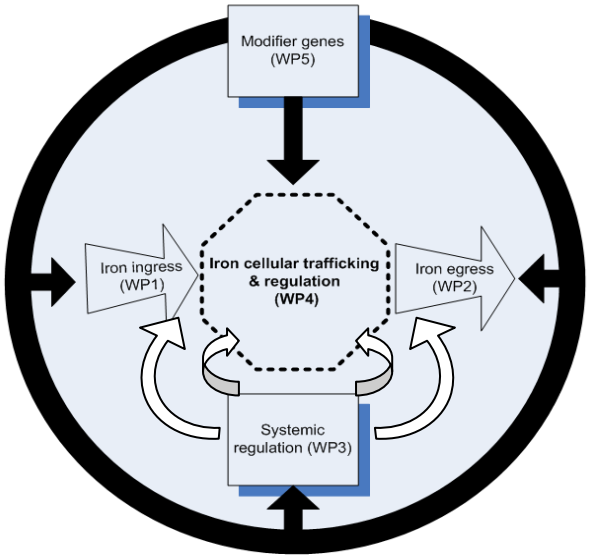
3. Improved mechanistic knowledge in iron-related disorders:

. Heme iron intestinal uptake is enhanced in knock-out (HFE/hepcidin) mouse models of hemochromatosis.
. Key role of local HFE expression in hepatocytes for iron overload development in HFE-related haemochromatosis
. Demonstration, in frataxin-deficient cells, that deferiprone restores cell functions impaired by frataxin deficiency (a model for Friedreich ataxia).
. Ferroportin mutations impair intracellular ferroportin trafficking and induce iron retetention in human monocytes.
. Importance of plasma hepcidin determination for differentiating anemia of chronic disease with or without iron deficiency anemia.

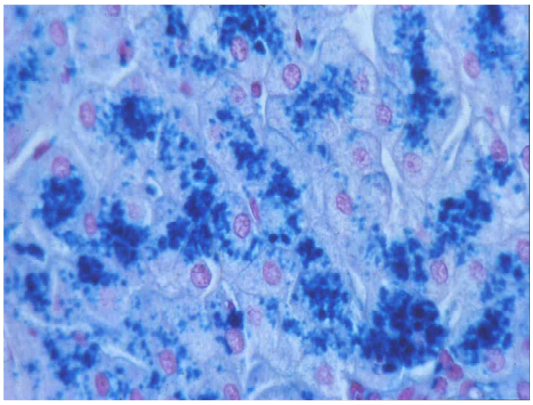
During the whole duration of the contract, the knowledge dissemination among the medico-scientific community has been the following : 58 original articles published in peer-reviewed Journals (among them: Nature Genetics, Science, Gastroenterology, Cell Metabolism, Blood), 36 general reviews, 63 invited conferences, 31 oral presentations, and 24 posters.

A.6 Diagrams, photos, logo, reference to website

Schematic presentation of the five scientific workpackages and of their interactions
The central octagonal box represents a cell.
The wide horizontal arrows represent the cellular ingress and egress of iron.
Grey plus white and white arrows indicate the potential targets of systemic regulation.
Black arrows indicate the potential targets of modifier genes.



Microscopic view of iron overloaded liver cells. Iron deposits are coloured in blue by Perls staining



EuroIron1 Logo

EuroIron1 Website

www.euroiron1.eu